HERC5 is an IFN-induced HECT-type E3 protein ligase that mediates type I IFN-induced ISGylation of protein targets

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Type I IFNs induce the expression of IFN-stimulated gene 15 (ISG15) and its conjugation to cellular targets. ISGylation is a multistep process involving IFN-inducible Ube1L, UbcH8, and a yet-to-be-identified E3 ligase. Here we report the identification of an IFN-induced HECT-type E3 protein ligase, HERC5/Ceb1, which mediates ISGylation. We also defined a number of proteins modified by ISG15 after IFN triggering or HERC5 overexpression. A reduction in endogenous HERC5 by small interfering RNA inhibition blocks the IFN-induced ISG15 conjugation. Conversely, HERC5 coexpression with Ube1L and UbcH8 induces the ISG15 conjugation in vivo independent of IFN stimulation. A targeted substitution of Cys-994 to Ala in the HECT domain of HERC5 completely abrogates its E3 protein ligase activity. Therefore, this study demonstrates that HERC5/Ceb1 is involved in the conjugation of ISG15 to cellular proteins.

ISG15 | Ceb1 | innate immunity | antiviral proteins

Type I IFNs (IFN-α/β) play an essential role in both innate antiviral and adaptive immune responses and are rapidly produced in response to microbial infection (1–3). They exert signals through the activation of the Janus kinase–signal transducer and activator of transcription pathway that mediates rapid induction of IFN-stimulated genes (ISGs) (4, 5). ISG15 is one of the most strongly induced genes after IFN treatment (6, 7) and is also significantly induced by viral infection (8, 9) and LPS treatment (10, 11). The ISG15 protein starts with two ubiquitin-related domains that have 27% sequence identity to ubiquitin and terminate in a conserved LRRG152 ubiquitin C-terminal motif. This study suggests that ISG15 could act in a similar way to ubiquitin and other ubiquitin-like proteins such as SUMO by forming an isopeptide bond with cellular proteins (6, 12, 13). The crystal structure of ISG15 revealed that ISG15 consists of two domains with ubiquitin-like folds joined by a linker sequence (14).

Conjugation of ISG15 to cellular proteins occurs in a parallel but distinct mechanism to that of ubiquitin (15–17). The E1 enzyme for ISG15, Ube1L, is a single-subunit enzyme and is identified in vitro by its ability to catalyze the formation of a thioester bond between ISG15 and Ube1L (17, 18). The Ube1L protein is highly similar to the E1 enzyme for ubiquitin at the protein level. However, this protein does not form a conjugate with ubiquitin, indicating that Ube1L is an E1 enzyme for the ISG15 conjugation system (ISGylation). Influenza virus blocks protein ISGylation by inhibiting the activation step through the interaction of the NS1B viral protein with ISG15 (18). This finding was the first suggestion that ISGylation might be important for protecting cells from viral infection.

Two groups recently found that a member of the ubiquitin E2-conjugating enzyme family, UbcH8, is involved in the ISGylation (19, 20). Like ISG15 and Ube1L, the expression of UbcH8 is also induced by IFN (21). The suppression of UbcH8 protein expression by RNA interference is shown to dramatically inhibit the total level of IFN-induced conjugation of ISG15 to cellular proteins (19, 20).

In the ubiquitin and ubiquitin-like proteins (ubls) system, E3 enzymes play a critical role in transferring ubiquitin or ubls such as SUMO from the E2-conjugating enzyme to a specific substrate. The substrate protein interacts directly with a specific domain in the E3 enzymes or through an adaptor associated with the E3 enzyme (22). E3 enzymes are known to be divided into two groups: HECT (homologous to E6-AP C terminus) and RING (really interesting new genes) proteins (22–25). Whereas RING-type proteins transfer ubiquitin directly from an E2 to a target, the HECT-type protein forms a thioester bond with ubiquitin or ubls by its active cysteine residue before transferring it to a substrate (25). The identity of the E3 enzyme(s) in the ISG15 conjugation system has not been resolved.

Here we report the identification of target proteins conjugated with ISG15 and present data supporting the contention that the covalent modification of cellular proteins by ISG15 is regulated by an IFN-inducible HECT domain-containing E3 protein ligase, HERC5, which itself is also a target for modification by ISG15. HERC5 catalyzes ISGylation by means of a catalytic cysteine residue at position 994 in the HECT domain. Moreover, HERC5 is found to be sufficient for inducing ISGylation in the absence of IFN. Knock-down of HERC5 by small interfering RNA (siRNA) results in the inhibition of IFN-induced ISG15 conjugation. Thus, HERC5 is an IFN-induced E3 protein ligase that mediates ISGylation.

Results

Identification and Purification of FLAG-ISG15-Associated and/or -Modified Proteins. To search for potential E3 enzyme(s) as well as proteins that were physically associated or covalently conjugated with ISG15, we generated A549 cell lines stably expressing ISG15 with two copies of FLAG tag (Fig. 1A). The FLAG tags were inserted at the N terminus of ISG15 because of the involvement of the conserved C terminus diglycine motif in conjugation with cellular proteins (Fig. 1A). The FLAG–ISG15 retains its conjugation activity to cellular proteins after 48 h of IFN-β treatment as compared with untreated control (Fig. 7A, which is published as supporting information on the PNAS web site). These data indicate that FLAG tags inserted at the N terminus of ISG15 do not disrupt the conjugation to cellular proteins after IFN-β treatment.

We next carried out large-scale isolation of ISG15-associated/
conjugated proteins by anti-FLAG immunopurification (Fig. 7B). Control A549 fractions were also analyzed to identify nonconjugated, covalently modified proteins. A total of 174 unique conjugated proteins, not found in control fractions, and represented by at least two peptides in tandem MS analyses, were identified. The ISG15 target proteins that fulfilled these stringent criteria are listed in Table 1, which is published as supporting information on the PNAS web site.

Twenty-six target proteins were selected for further validation of their conjugation or association with ISG15. Twenty-four of 26 putative ISG15 target proteins were found to form conjugates with ISG15 after IFN-β treatment (Table 1, bold). Two other ISG15-copurified proteins, Hsp70 and pICln, were not directly conjugated to ISG15 but formed a specific, noncovalent interaction (data not shown). Of the proteins that were substrates for ISG15 conjugation, a number, such as destrin and cofilin, were found to conjugate with a single ISG15 as they migrated at the expected molecular masses for destrin*FLAG–ISG15 (M, ~37 kDa) (Fig. 1B, lane 2) and cofilin*FLAG–ISG15 (M, ~37 kDa) (Fig. 1C, lane 2). On the other hand, some proteins, such as enolase and GAPDH, existed as multiply conjugated species that were possibly modified by one or more ISG15s (Fig. 1D and E, lane 2). At least two isoforms of enolase-conjugated species were observed, migrating at the positions expected for one and two ISG15s (Fig. 1D, lane 2). Similar to enolase, GAPDH also displayed a number of conjugated species that migrated at the positions expected for one and two ISG15s in SDS/PAGE (Fig. 1E, lane 2). In addition to conjugation, we also observed noncovalent association between ISG15 and cellular substrates (Fig. 1B–E, shown by arrowhead). Thus, we have identified a large number of cellular proteins that form either single or multiple conjugation and noncovalent interactions with ISG15.

HERC5/Ceb1 Is Copurified with ISG15, and Its Expression Is Regulated by Type I IFN. Conjugation of ISG15 with cellular proteins is catalyzed by the E1 activating enzyme Ube1L and the E2-conjugating enzyme UbcH8 (17). Thus far, no E3 enzyme(s) has been identified. In ubiquitin system, E3 enzymes, with either a HECT domain or a RING domain, play a critical role in recruiting the ubiquitin-loaded E2, recognizing specific substrates and facilitating the transfer of the ubiquitin from the E2 to the lysine residue in the substrates (22). Among all ISG15-associated/conjugated candidate proteins, we noticed that a protein known as HERC5 (or Ceb1) contains a HECT-type domain at its C terminus (Fig. 8, which is published as supporting information on the PNAS web site) (26). As shown in Table 2, this is published as supporting information on the PNAS web site, HERC5 was represented by five individual peptides from tandem MS sequencing and was not found in the control sample, indicating that this protein is specifically copurified with FLAG–ISG15.

The primary sequence of HERC5 revealed the presence of RCC1-like (regulator of chromosome condensation-1) and HECT domains that span residues 209–258 and 676-1024, respectively (Fig. 8A) (26). The HECT domain was previously demonstrated to interact with a ubiquitin-conjugating enzyme (23). Within the HECT domain of HERC5, a conserved cysteine residue residing in all known HECT-type protein ligases was also found and located at position 994 (Fig. 8B) (26). Because HERC5/Ceb1 possesses all of the unique structural features of a mammalian E3 protein ligase, we hypothesized that HERC5 functions as an E3 protein ligase in ISGylation.

Because HERC5 is identified by the pulling down of ISGylated proteins and ISG15-associated proteins, we first determined whether ISG15 binds HERC5 or forms covalent conjugated species with HERC5. HeLa cells were cotransfected with FLAG–HERC5 and Myc–ISG15 by anti-Myc and anti-FLAG Western blotting, respectively. Moreover, we saw free forms of HERC5 or anti-FLAG Western blotting to determine whether higher-molecular-weight forms of HERC5 or free forms of ISG15 could be detected. As shown in Fig. 2A, we saw multiple species of high-molecular forms of HERC5 in IFN-β-treated cells expressing both FLAG–HERC5 and Myc–ISG15 by anti-Myc and anti-FLAG Western blotting, respectively. Moreover, we saw free forms of ISG15 copurified with HERC5 in both untreated and IFN-β-treated cells (Fig. 2A Left). These data indicate that HERC5 physically associates with ISG15 and itself is a target for modification by ISG5.

We then sought to examine whether type 1 IFN induces HERC5 mRNA expression in both HeLa and A549 cell lines because the expression of Ube1L and UbcH8 has previously been shown to be induced by IFN-β (21). Both HeLa and A549 cells were treated with IFN for 6, 12, 24, and 48 h. As shown in Fig. 2B, we detected basal levels of HERC5 mRNA in both untreated HeLa (Left) and A549 (Right) cells. Upon IFN-β stimulation, HERC5 mRNA was rapidly induced in both cell lines after 6 h (Fig. 2B). At 12 h there were ~30-fold increments in HERC5 mRNA expression levels compared with their respective untreated controls for both HeLa and A549 cells (Fig. 2B). The mRNA for HERC5 was continuously expressed at high levels at 24 and 48 h after IFN-β treatment as compared with...
Coexpression of HERC5 with Ube1L and UbcH8 Restores ISG15 Conjugates in Vivo Without Type I IFN for Induction. Because HERC5 expression is induced by IFN, we tested whether overexpression of HERC5 in combination with Ube1L and UbcH8 would lead to ISGylation in the absence of IFN-β. HeLa cells transfected with either Ube1L (Fig. 4A, lane 2) or UbcH8 (Fig. 4A, lane 3) expressed no ISG15 conjugates. A partial expression of ISG15 conjugates was observed in HeLa cells transfected with Ube1L and UbcH8 (Fig. 4A, lane 4), which may have been because of a low level of HERC5 expression in HeLa cells (refer to RNA work in Fig. 2B Left). When HERC5 is overexpressed, the production of ISG15 conjugates by HeLa cells is dramatically enhanced (Fig. 4A, lanes 5). These data strongly implicated HERC5 as an E3 protein ligase whose overexpression is sufficient to drive ISGylation.

Next we examined a number of proteins known to be ISGylated after IFN-β treatment, for conjugation with ISG5 in HeLa cells overexpressing HERC5. HeLa cells were transfected with FLAG–ISG5 alone (Fig. 4B–I, lane 1), FLAG–ISG5 plus vectors expressing Ube1L and UbcH8 (Fig. 4B–I, lane 2), or HERC5, Ube1L, and UbcH8 (Fig. 4B–I, lane 3). Cells were harvested after 48 h. FLAG–ISG5 conjugates were immunoprecipitated with anti-FLAG antibody. The protein complexes were separated in SDS/PAGE and detected with specific antibodies. All of the target proteins examined were found to be covalently conjugated with ISG5 when HERC5 is overexpressed (Fig. 4B–I, lanes 3). Three target proteins, destrin, enolase, and GAPDH, were observed to have low levels of conjugation in HeLa cells transfected with Ube1L and UbcH8 alone (Fig. 4B, D, and E, lane 2). However, their levels of conjugation were dramatically enhanced in cells transfected with HERC5, Ube1L, and UbcH8 (Fig. 4B, D, and E, lane 3). Interestingly, the presence of multiple conjugated species for both enolase and GAPDH were also observed after HERC5 overexpression, indicating that HERC5 is essential for both...
HERC5 is not dependent on type I IFNs. Upon expression of HERC5, the enzymatic activity of single and multiple conjugations in target proteins require the presence of HERC5, which functions as an E3 protein ligase involved in ISGylation. Both HERC5 and Ube1L and UbcH8 were detected by antibodies against Ube1L (C Upper) and UbcH8 (C Lower), respectively. (D) Suppression of IFN-β-induced HERC5 mRNA expression in HeLa cells constitutively expressing shRNA–HERC5–1-4. Efficiency of shRNA–HERC5–1-4 in inhibition of endogenous HERC5 RNA expression was determined by using real-time PCR with specific primers for HERC5. (C) HERC5 elimination has no effect on the induction of Ube1L and UbcH8 by type I IFN. Total lysates were prepared from A549 cells transfected with control siRNA (lane 1) or siRNAs against HERC5 (lanes 2 and 3) at 24 h after IFN-β treatment. The levels of protein expression of Ube1L and UbcH8 were detected by antibodies against Ube1L (C Upper) and UbcH8 (C Lower), respectively. (D) Suppression of IFN-β-induced HERC5 mRNA expression in HeLa cells constitutively expressing shRNA–HERC5–1-4. Efficiency of shRNA–HERC5–1-4 in inhibition of endogenous HERC5 RNA expression was determined by using real-time PCR with HERC5-specific primers. (E) Inhibition of IFN-β-induced HERC5 conjugation in HeLa cells stably expressing shRNA against HERC5. HeLa cells were stably transfected with vectors expressing shRNA–HERC5–1-4 and selected with 1 μg/ml puromycin. Cells were treated with IFN-β for 48 h, and ISG15 conjugates were analyzed by immunoblotting with anti-ISG15 antibody.

**Cys-994 Within the HECT Domain of HERC5 Is Essential for Its Protein Ligase Activity in ISGylation.** All HECT-type ubiquitin protein ligases contain a conserved active cysteine residue that forms an intermediate bond with ubiquitin before transferring it to the substrates (Fig. 8B). To further demonstrate that HERC5 is an E3 protein ligase for ISGylation, the putative active cysteine residue (C994), based on conservation with other HECT-domain-containing E3s, was mutated to an alanine residue by site-specific mutagenesis. HeLa cells were cotransfected with Myc–ISG15 and either wild-type FLAG–HERC5 (Fig. 5, lane 4) or mutant FLAG–HERC5 (Fig. 5, lane 5). This point mutation in the cysteine residue (C994) completely abolishes its E3 activity in ISGylation (Fig. 5 Upper, compare lanes 4 and 5). The expression levels of wild-type and mutant HERC5 proteins were found to be comparable (Fig. 5 Lower). These data demonstrate that HERC5 acts as an E3 protein ligase in ISGylation by means of a conserved cysteine residue, C994, found in the HECT domain.

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**Fig. 3.** Depletion of HERC5 inhibits the induction of ISG15 conjugation by IFN-β. (A) HERC5 depletion by siRNAs inhibits ISG15 conjugation induced by IFN-β in A549 cells. The A549 cells were transfected with control siRNA (lane 1) or siRNAs against HERC5 (lanes 2 and 3). Twenty-four hours after transfection, total lysates were harvested and separated in SDS/PAGE. ISG15 conjugates were detected by anti-ISG15 antibody. (B) Suppression of HERC5 mRNA expression in A549 cells by siRNAs. Efficiency of siRNA–HERC5–I and siRNA–HERC5–II in inhibition of HERC5 RNA expression was determined in A549 cells transfected with siRNA control and siRNAs for HERC5 24 h after IFN-β treatment. Expression of HERC5 mRNA was analyzed by real-time PCR with specific primers for HERC5. (C) HERC5 elimination has no effect on the induction of Ube1L and UbcH8 by type I IFN. Total lysates were prepared from A549 cells transfected with control siRNA (lane 1) or siRNAs against HERC5 (lanes 2 and 3) at 24 h after IFN-β treatment. The levels of protein expression of Ube1L and UbcH8 were detected by antibodies against Ube1L (C Upper) and UbcH8 (C Lower), respectively. (D) Suppression of IFN-β-induced HERC5 mRNA expression in HeLa cells constitutively expressing shRNA–HERC5–1-4. Efficiency of shRNA–HERC5–1-4 in inhibition of endogenous HERC5 RNA expression was determined by using real-time PCR with HERC5-specific primers. (E) Inhibition of IFN-β-induced HERC5 conjugation in HeLa cells stably expressing shRNA against HERC5. HeLa cells were stably transfected with vectors expressing shRNA–HERC5–1-4 and selected with 1 μg/ml puromycin. Cells were treated with IFN-β for 48 h, and ISG15 conjugates were analyzed by immunoblotting with anti-ISG15 antibody.

**Fig. 4.** HERC5, together with Ube1L and UbcH8, induces the FLAG–ISG15 conjugation in HeLa cells without type I IFN treatment. (A) HeLa cells were transfected with expression vector encoding for FLAG–ISG15 plus vectors encoding Ube1L (lane 2), UbcH8 (lane 3), Ube1L and UbcH8 (lane 4), or Ube1L, UbcH8, and HERC5 (lanes 5). Twenty-four hours after transfection, total lysates were prepared, and FLAG–ISG15 conjugates were detected with anti-FLAG antibody. (B–I) HeLa cells were transfected with expression vector encoding for FLAG–ISG15 (lane 1), FLAG–ISG15 plus vector encoding Ube1L and UbcH8 (lane 2), or FLAG–ISG15 construct together with vectors encoding Ube1L, UbcH8, and HERC5 (lanes 3). Total lysates were harvested, FLAG–ISG15 conjugates were immunoprecipitated with anti-FLAG M2-conjugated agarose, and protein complexes were separated in SDS/PAGE and then immunoblotted with antibodies against destrin (B), cofilin (C), enolase (D), GAPDH (E), Hsp27 (F), Hsc70 (G), peroxiredoxin I (H), and peroxiredoxin VI (I).
HERC5 interacts with ISGylated substrates, HSC70, and Thioredoxin Reductase. To further support that HERC5 is a protein ligase for ISG15, we next tested whether HERC5 interacts with ISGylated substrates, Hsc70, and thioredoxin reductase. HEla cells were transfected with FLAG–HERC5 (Fig. 6, lane 3), ISGylated substrate (Fig. 6, lane 2), FLAG–HERC5 plus HA-Hsc70 (Fig. 6, lane 4) or HA–thioredoxin reductase (Fig. 6B, lane 4). Cell extracts were immunoprecipitated with an anti-FLAG monocular antibody. The immunoprecipitates were then subjected to SDS/PAGE and immunoblotted with antibodies against FLAG or hemagglutinin (HA). As shown in Fig. 6, the antibody against FLAG precipitated FLAG–HERC5 in cells expressing FLAG–HERC5 (Fig. 6 Middle, lanes 3 and 4), but not in the control cells (Fig. 6 Middle, lanes 1 and 2). The FLAG antibody also precipitated Hsc70 (Fig. 6A Top, lane 4) or thioredoxin reductase (Fig. 6B Top, lane 4). The expression of cellular substrates was the same by immunoblotting cell lysates with an HA antibody (Fig. 6 Bottom, lanes 2 and 4). These results suggest that HERC5 acts as an E3 protein ligase for ISGylation by recruiting cellular substrates for modification by ISG15, which is also found to be associated with HERC5 (Fig. 2A).

Discussion
HERC5 is an IFN-Inducible Protein Ligase for ISG15 Conjugation System. We have described the properties of HERC5 identified through its interaction with ISG15. This interaction was detected by using an affinity-based purification scheme involving an antibody against FLAG–ISG15 in A549 cells treated with IFN-β. HERC5 is a member of a group of related proteins known as the HERC family (27, 28). The human HERC family currently consists of six members, HERC1 to HERC6, and is characterized by the presence of a HECT domain and one or more RCC1-like domains (RLDs). Proteins with a HECT domain are known to act as E3 protein ligases for ubiquitin and ubiquitin-like proteins, such as E6-AP and Nedd4 (29). Here we have provided evidence suggesting that HERC5 is an IFN-inducible E3 protein ligase that is required and sufficient for the ISG15 conjugation system in vivo. First, a knock-down of HERC5 expression by RNA interference technology completely inhibited the induction of ISG15 conjugation induced by IFN. Second, HERC5 coexpression with the Ube1L and UbcH8 suffices to mediate ISG15 conjugation in vivo even if in the absence of IFN treatment. Third, a mutation of a conserved cysteine residue in the HECT domain of HERC5 to an alanine residue abolished its protein ligase activity in vivo. This result is consistent with the hypothesis that C994 is important for transferring ISG15 from UbcH8 to specific target proteins. Fourth, HERC5 interacts with both ISG15 and ISGylated substrates such as Hsc70 and thioredoxin reductase.

HERC5 will be needed to provide insight into the specificity of ISGylation in this study was also demonstrated by an independent group while this article was under review (30). One of the challenging tasks is to express HERC5 for promotion of ISGylation in vitro; however, we and Dastur et al. (30) had some difficulty expressing either the full-length or the HECT domain of HERC5 in the bacterial system, and this issue remains to be resolved. The N-terminal region of HERC5 possesses at least one RLD. RLDs have been shown to interact with several proteins, and it is possible that the RLDs serve as the regions for recruiting specific protein substrates. In addition, the middle region, between RLDs and the HECT domain of HERC5, displays no obvious homology to any known proteins. This region may also serve a role in recognizing target proteins. Further structure–function studies in HERC5 will be needed to provide insight into the specificity of HERC5 in recognizing its target proteins.

Target Proteins Modified by HERC5. By using a combination of affinity purification and MS, we identified 174 candidate proteins that were covalently conjugated or interacted with ISG15 upon IFN treatment. Of 27 target proteins examined, 24 were identified to conjugate with ISG15, and the other three proteins were found to interact with ISG15 (Table 1 and data not shown). Given that most of the candidates we chose to pursue were modified by the ISG15 conjugation pathway, we are highly confident that our large data set represents bona fide substrates for ISG15 in vivo.

Eight putative IFN-inducible ISG15 targets (Mxa, IFIT1, signal transducer and activator of transcription 1, Ube1L, tryptophanyl tRNA synthetase, TRIM21, gelsolin, and HERC5) are identified (4, 5). We chose four of the IFN-induced proteins (Mxa, IFIT1, signal transducer and activator of transcription 1, and tryptophanyl tRNA synthetase) and verified that they are ISG15-conjugated in
Expression Constructs. Generation of FLAG–ISG15, Myc–ISG15, UbcH8, Ube1L, and HERC5. These proteins are 14-3-3\textsuperscript{eral} proteins identified in our screening but not in the other two candidates identified in our study, only 61 proteins were found U937- or UBP43-deficient mouse embryonic fibroblasts (34, 35). Of the approach to identify ISG15-modified proteins from HeLa cells and system, two groups independently reported using a proteomic technique in the presence of HERC5 overexpression. The ISGylation of the identified protein targets was observed after type I IFN treatment or in the presence of HERC5 overexpression.

Materials and Methods

Generation of FLAG–ISG15, Myc–ISG15, UbcH8, Ube1L, and HERC5 Expression Constructs. cDNA encoding for ISG15 was amplified from a human splenic cDNA library purchased from CLONTECH. Either two copies of FLAG tag or three copies of Myc tag were inserted at the N terminus of ISG15 and subcloned into pQXIX expression vector (CLONTECH). Ube1L cDNA was inserted at the N terminus of ISG15 and subcloned into pQXIX vector (CLONTECH). UbcH8 was also cloned by PCR into pLNCX2 expression vector (CLONTECH). HERC5, which acts as an ISG15 E3 protein ligase. This activity is strictly dependent on a conserved Cys residue at position 12. Ritchie, K. J. & Zhang, D. E. (2004) Nat. Rev. Immunol. 5, 675–687.


Generation of A549 Cells Stably Expressing FLAG–ISG15. A549 cells were transfected with expression construct encoding FLAG–ISG15 by Lipofectamine 2000 (Invitrogen). Cells were transfected by selecting 500 units/ml IFN-\beta. Cells were lysed in 200 ml of 1% Triton X-100 in 10 ml Tris and 150 ml NaCl (pH 7.4) (TBST) on ice for 1 h. After removing nuclei and cell debris by centrifugation, crude lysate was first subjected to a mouse IgG-conjugated agarose column (Sigma) followed by an anti-FLAG M2-conjugated agarose column (Sigma) at 4°C, respectively. ISG15-associated and/or-modified proteins were eluted with 3.5 M MgCl\textsubscript{2} after washing extensively with 1% TBST buffer. Portions of the fractions collected were separated by SDS/PAGE and were further detected by using an anti-FLAG antibody. Fractions containing FLAG–ISG15 were pooled and concentrated by using Centricon (Amicon).

MS Analysis. MS analysis was performed in-house at the proteomic facility of the Genome Institute of Singapore. In brief, the samples were reduced and alkylated with iodoacetamide, digested with trypsin, and subjected to LC-MS/MS analysis on an LCQ Deca Plus ion-trap mass spectrometer. Peptide masses were queried against entries in the International Protein Index human database by using Mascot (Matrix Science). For a protein to be considered as a “hit,” a minimum of two matching peptides was required.

Verification of ISG15 Target Proteins. HeLa cells were plated on 100-mm dishes (2.2 × 10\textsuperscript{5}) and transfected using Lipofectamine 2000. After 4 h, dishes were washed and replaced with complete DMEM-H media either with or without IFN-\beta (500 units/ml). The cells were harvested after 48 h. Total lysates were extracted for Western blotting or immunoprecipitation with anti-FLAG antibody. The sequences of siRNAs and antibodies used can be found in Fig. 10. For more information, see Supporting Materials and Methods, which is published as supporting information on the PNAS web site.

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